OHIO AGRICULTURAL RESEARCH AND DEVELOPMENT CENTER

Bioabatement to remove microbial inhibitors from *Miscanthus giganteus* hydrolysates for enhanced butanol fermentation

Chidozie V. Agu, Victor C. Ujor, and Thaddeus C. Ezeji

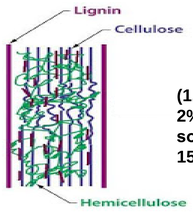
Abstract

The recalcitrant nature of cheap lignocellulose warrants pretreatment process to disrupt the lignin matrix and expose the carbohydrate fraction to enzymatic saccharification. Generation of **lignocellulose-derived microbial inhibitory compounds (LDMICs)** during the pretreatment process undermines large-scale utilization of biomass for biofuel (e.g. **butanol**) production. LDMICs are derived from lignin (e.g. vanillin), cellulose (e.g. 5-hydroxymethylfurfural [HMF]), and hemicellulose (e.g. acetic acid) fractions of lignocellulose. These compounds impair butanol fermentation by disrupting the growth of butanol-producing *Clostridium beijerinckii* through diverse mechanisms including perturbation of redox and energy state of the cell, inhibition of glycolytic enzymes, and damage to cell membrane, nucleic acids and organelles. Although LDMICs can be removed from lignocellulosic biomass hydrolysates (LBH) by physicochemical methods, these methods increase the overall butanol production cost. **Bioabatement**, a cost-effective alternative, employs microorganisms that selectively metabolize LDMICs in the presence of fermentable sugars. In this study, we demonstrate the ability of the bacterium, *Cupriavidus basilensis* ATCC®BAA-699 to metabolize pure LDMICs and *Miscanthus giganteus* biomass hydrolysate (MH)-associated LDMICs. Notably, MH was generated by dilute-acid (2% H_2SO_4) pretreatment at 15% biomass solids loading in a reactor at 180˚C and 150 psi for 1 h. The hydrolysate was then detoxified by *C. basilensis* prior to enzymatic hydrolysis to release fermentable sugars. Acetone-butanol-ethanol (**ABE**) fermentation of ***C. basilensis*-detoxified MH** resulted in ~70% increase in ABE concentration when compared to the non-detoxified control. These results underscore the feasibility of biological removal of LDMICs from pre-enzyme hydrolyzed LBH prior to fermentation to butanol.

Introduction

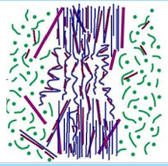
Recalcitrant biomass

A.



(1.) Pretreatment: 2% sulfuric acid; 15% solids loading; 180˚C, 150 psi; 1 h

Depolymerized biomass



Oligosaccharides, disaccharides, monosaccharides, and LDMICs

(2.) Detoxification prior to enzymatic hydrolysis - addition of cellulases & xylanases

More monosaccharides; less LDMICs – Furans, phenolics, etc.

❑ Pretreatment process depolymerizes recalcitrant biomass to release sugars & inhibitors

B.

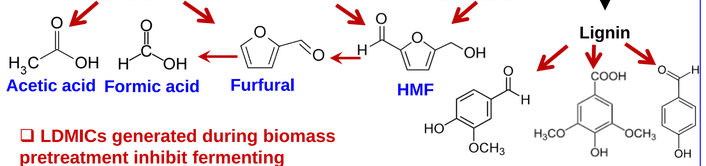
Pretreated biomass

Xylose and Arabinose from hemicellulose

Galactose and mannose from hemicellulose

Glucose mainly from cellulose

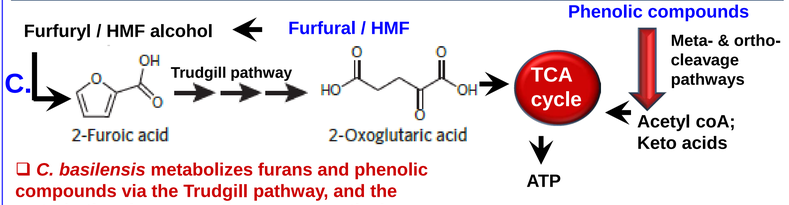
Lignin



❑ LDMICs generated during biomass pretreatment inhibit fermenting microbes e.g. *C. beijerinckii*

Phenolic compounds

Introduction (cont'd)



❑ *C. basilensis* metabolizes furans and phenolic compounds via the Trudgill pathway, and the meta-/ortho-cleavage pathways, respectively

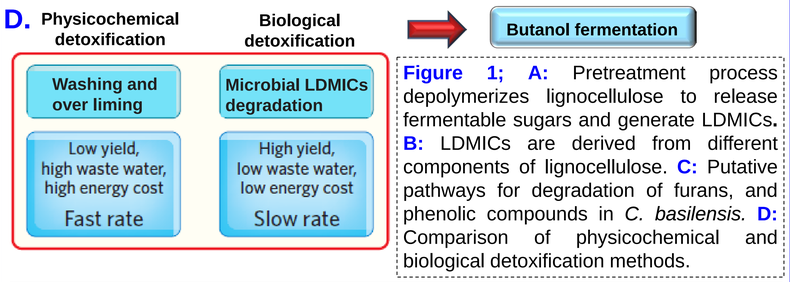


Figure 1; A: Pretreatment process depolymerizes lignocellulose to release fermentable sugars and generate LDMICs**. B:** LDMICs are derived from different components of lignocellulose. **C:** Putative pathways for degradation of furans, and phenolic compounds in *C. basilensis.* **D:** Comparison of physicochemical and biological detoxification methods.

Specific aims

- To detoxify *Miscanthus giganteus* lignocellulosic biomass hydrolysates (MH) using *Cupriavidus basilensis* ATCC®BAA-699.
- To evaluate the fermentability of detoxified and non-detoxified MH hydrolysates.

Materials and methods

Biomass pretreatment reactor: 15% solids loading; 2% sulfuric acid; 180˚C, 150 psi; 1 h

Figure 2

Biodetoxification of MH

MH

Inoculum

100-ml MH + seed culture. Shake-flask incubation @ 144 rpm, 25ºC for only 12 h to minimize sugar loss

Furfural-adapted *C. basilensis* seed culture

Analyses

Enzymatic hydrolysis: more fermentable sugars

Plate count

Determination of cell growth ($OD_{600\,nm}$)

Butanol fermentation

Detoxified MH

Non-detoxified MH (control)

100%, 80%, and 60% MH + glucose addition to 60 g/L total sugar + P2 stock solution + 6% *C. beijerinckii* NCIMB 8052 pre-culture

Sample collection: 12 hourly for Analyses

Gas chromatography: Analysis of butanol, acetone, ethanol, acetic & butyric acids

HPLC: Quantification of sugars & LDMICs

Results

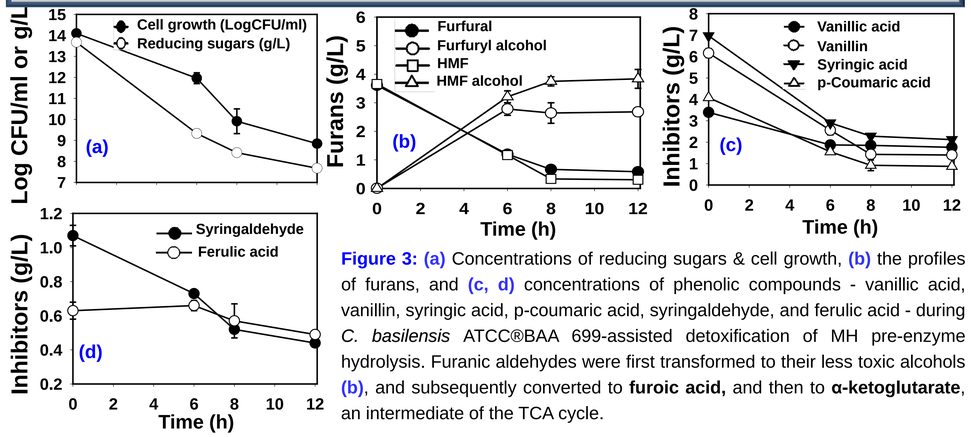


Figure 3: **(a)** Concentrations of reducing sugars & cell growth, **(b)** the profiles of furans, and **(c, d)** concentrations of phenolic compounds - vanillic acid, vanillin, syringic acid, p-coumaric acid, syringaldehyde, and ferulic acid - during *C. basilensis* ATCC®BAA 699-assisted detoxification of MH pre-enzyme hydrolysis. Furanic aldehydes were first transformed to their less toxic alcohols **(b)**, and subsequently converted to **furoic acid,** and then to **α-ketoglutarate**, an intermediate of the TCA cycle.

In parallel, phenolic compounds were metabolized via the meta- and ortho-cleavage pathways to keto acids and acetyl coA - intermediates of the TCA cycle (**Fig. 1C**). Sugars were concomitantly utilized **(a)** along side furfural and HMF **(b)** by *C. basilensis.* Phenolic compounds in the MH were reduced to more than half their initial concentrations within 12 h **(c,d)**.

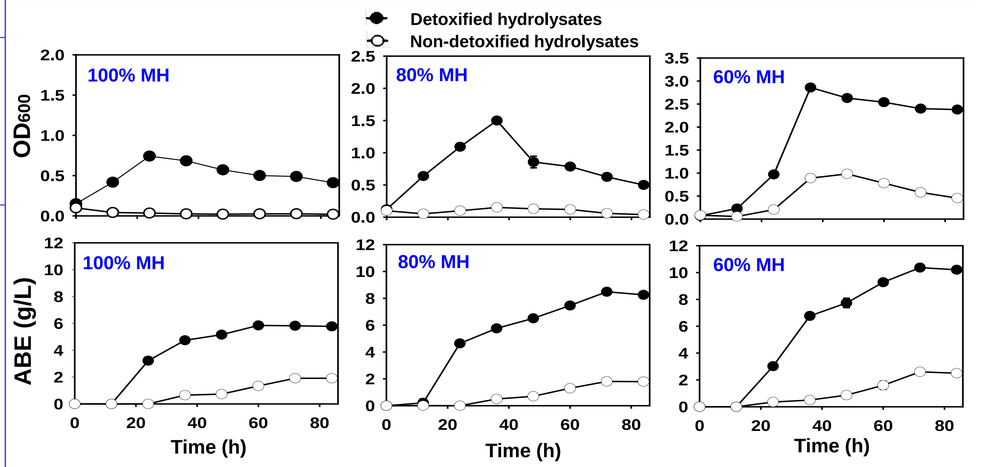


Figure 4: Cell growth and ABE concentration during fermentation of 100%, 80%, and 60% detoxified (tests) and non-detoxified (controls) MH, to butanol by *C. beijerinckii*. In all instances, cell growth and [ABE] for test cultures were significantly higher ($p<0.05$) when compared to the controls. Cell growth and [ABE] positively correlate with hydrolysate dilution due to decrease in [LDMICs]. Also, inefficient assimilation of acetic and butyric acids were observed for the controls, suggesting disruption of transition from acidogenesis to solventogenesis (*data not shown*).

Conclusions and Discussion

❖We demonstrate for the first time, the use of *C. basilensis* ATCC® BAA-699 to remove LDMICs from LBH and **enhance butanol fermentation.**

❖These results substantiate the hypothesis that *C. basilensis* ATCC® BAA-699, which grows on pure LDMICs as sole carbon substrate(s), can be used to detoxify biomass hydrolysates for enhanced butanol fermentation.

❖Loss of fermentable sugars due to co-utilization of LDMICs and sugars by this bacterium was minimized by adopting a bioabatement design wherein pretreated biomass was first detoxified **(< 12 h**) prior to enzymatic hydrolysis.

❖Pretreatment conditions in which less glucose is generated will further reduce loss of sugars to *C. basilensis* during biological abatement of LDMICs.

Acknowledgements

❖ We are grateful to Christopher Okonkwo and Shomaila Sikandar for their support in the lab.

❖ Financial supports from Ohio Plant Biotechnology Consortium (OPBC), Ohio Agricultural Research and Development Center (OARDC), and the Hatch grant (Project No. OHO01333) are acknowledged.

References

❖ Dong H, and Bao J. (2010) Biofuel via biodetoxification. *Nature Chemical Biology*, **6**(5): 316-318.

❖ Koopman F, Wierckx N, de Winde JH, and Ruijssenaars HJ (2010) Identification and characterization of the furfural and 5-(hydroxymethyl)furfural degradation pathways of *Cupriavidus basilensis* HMF14. *Proceedings of National Academy of Science* ,USA, 107(11): 4919 – 4924.

❖ Steinle P, Stucki G, Steller R, and Hanselmann KW. (1998) Aerobic mineralisation of 2,6-dichlorophenol by *Ralstonia sp*. Strain RK1. *Applied and Environmental Microbiology.* **64**(7):2566

❖http://www.purdue.edu/lorre/research/bioenergy/index.php.

THE OHIO STATE UNIVERSITY
COLLEGE OF FOOD, AGRICULTURAL, AND ENVIRONMENTAL SCIENCES

DEPARTMENT OF ANIMAL SCIENCES, THE OHIO STATE UNIVERSITY & OARDC, 1680 MADISON AVENUE, WOOSTER, OH, 44691